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Phosphate transport across biomembranes and cytosolic phosphate homeostasis in barley leaves*

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Abstract. Barley (*Hordeum vulgare* L.) plants were grown hydroponically with or without inorganic phosphate (P_i) in the medium. Leaves were analyzed for the intercellular and the intracellular distribution of P_i. Most of the leaf P_i was contained in mesophyll cells; P_i concentrations were low in the xylem sap, the apoplast and in the cells of the epidermis. The vacuolar concentration of P_i in mesophyll cells depended on P_i availability in the nutrient medium. After infiltrating the intercellular space of leaves with solutions containing P_i, P_i was taken up by the mesophyll at rates higher than 2.5 $\mu mol \cdot (g\ fresh\ weight)^{-1} \cdot h^{-1}$. Isolated mesophyll protoplasts did not possess a comparable capacity to take up P_i from the medium. Phosphate uptake by mesophyll protoplasts showed a biphasic dependence on P_i concentration. Uptake of P_i by P_i-deficient cells was faster than uptake by cells which had P_i stored in their vacuoles, although cytoplasmic P_i concentrations were comparable. Phosphate transport into isolated mesophyll vacuoles was dependent on their P_i content; it was stimulated by ATP. In contrast to the vacuolar P_i concentration, and despite different kinetic characteristics of the uptake systems for P_i of the plasmalemma and the tonoplast, the cytoplasmic P_i concentration was regulated in mesophyll cells within narrow limits under very different conditions of P_i availability in the nutrient medium, whereas vacuolar P_i concentrations varied within wide limits.

Key words: Apoplast – Cytosol – *Hordeum* (phosphate homeostasis) – Phosphate transport – Plasmalemma – Tonoplast

Introduction

For adequate growth, plants require approximately one molecule of inorganic phosphate (P_i) for every 500 molecules of carbon fixed in photosynthesis (Marschner 1986). Phosphate is a constituent of nucleic acids and phospholipids, and is also of prime importance in energy metabolism. Phosphate deficiency rapidly reduces plant growth; it decreases photosynthesis and changes assimilate partitioning (Dietz 1989; Sicher and Kremer 1988). Within the cells, a metabolically active P_i pool can be distinguished from an inactive P_i pool (Ullrich et al. 1965; see Bieleski 1973 for a review; Rebeille et al. 1983). Phosphate is exchanged between both pools only slowly, depending on metabolic demands (Ullrich et al. 1965; Foyer and Spencer 1986). Whereas the ^{31}P-nuclear magnetic resonance (NMR) technique is capable of distinguishing two P_i pools in leaves, it does not differentiate between P_i contained in the cytoplasmic organelles and in the cytosol. Also, the kinetics of P_i transport cannot be determined with this technique (see Roberts 1984 for a review). Studies with isolated organelles allow the investigation of the intracellular compartmentation and transport of P_i. However, P_i may leak from organelles. Cross contamination of organelles, particularly by vacuolar material, results in an overestimation of P_i when non-aqueous techniques are employed (Dietz and Heber 1984).

This communication describes effects of P_i nutrition on the P_i status of barley leaves. Vacuoles can rapidly be isolated from protoplasts. Therefore, isolated vacuoles are used in this communication to distinguish between cytoplasmic and vacuolar P_i levels. This is possible as the tonoplast of isolated vacuoles has a low permeability to P_i (Martinoia et al. 1986). We also investigate the role of the apoplast and of the vacuole in maintaining the P_i homeostasis of the cytoplasm of leaf cells.

Material and methods

Plant growth. Barley (*Hordeum vulgare* L., cv. Gerbel) was grown in hydroponic culture. The standard medium contained 9 $mmol \cdot l^{-1}$ KNO_3, 6 $mmol \cdot l^{-1}$ $Ca(NO_3)_2$, 3 $mmol \cdot l^{-1}$ $MgSO_4$, 1.5 $mmol \cdot l^{-1}$ KH_2PO_4, 0.13 $mmol \cdot l^{-1}$ Fe-ethylenediaminetetraacetate (EDTA) and micro nutrients. Plants used for the preparation of xylem sap grew in a medium which contained 5 $mmol \cdot l^{-1}$ KNO_3 and 4 $mmol \cdot l^{-1}$ KCl instead of 9 $mmol \cdot l^{-1}$ KNO_3. Levels

* Dedicated to Professor Wilhelm Simonis on the occasion of his 80th birthday

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of P_i were increased as indicated below to obtain plants with elevated P_i levels. Phosphate-deficient plants were grown in the absence of P_i. Ten days after sowing, the primary leaves and the seeds were removed from the plants to minimize transfer of endogenous P_i to young tissue.

Isolation of protoplasts and vacuoles. Mesophyll protoplasts were prepared from primary or secondary leaves 10 or 21–23 d, respectively, after sowing. The procedure was essentially as described by Kaiser et al. (1982), but sucrose and glycine betaine substituted for sorbitol and Percoll in the isolation media. The enzymes for protoplast isolation were freed from P_i by gel filtration.

Protoplasts from the cells of the epidermis had a low density and were separated from heavier mesophyll protoplasts by flotation. After complete digestion of the leaves, protoplasts from primary leaves of 10-d-old plants were resuspended in sorbitol medium (450 mmol·l^{-1} sorbitol; 20 mmol·l^{-1} 2-(N-morpholino)ethanesulfonic acid/KOH, pH 6.5; 20 mmol·l^{-1} potassium gluconate; 3 mmol·l^{-1} $CaCl_2$; 0.1% polyvinylpyrrolidone) and overlayered with glycine betaine medium (450 mmol·l^{-1} glycine betaine instead of sorbitol). A fraction consisting of the large protoplasts from the upper epidermis and of light mesophyll protoplasts was recovered from the interphase. The epidermal protoplasts were further purified by gradient centrifugation: sorbitol medium was added to the protoplast suspension (equal volumes) and overlayered with a mixture of glycine betaine medium and sorbitol medium (3:2, v/v) and then with glycine betaine medium. The gradient was spun at 600·*g* for 10 min. The pale, chlorophyll-less protoplast fraction was removed and Percoll was added to a final concentration of 10% (v/v). A mixture of sorbitol and glycine betaine medium (7:3, v/v) was layered on the suspension and glycine betaine medium added on top. The gradient was spun as above and the chlorophyll-free band of intact epidermal protoplasts was recovered. Protoplast numbers and volumes were determined by microscopic analysis. Intactness was demonstrated by accumulation of neutral red in the large central vacuole and by the ability of the protoplasts to incorporate [^{35}S]methionine into material which could be precipitated by trichloroacetic acid.

Vacuoles were isolated by the method of Martinoia et al. (1982). For measuring P_i uptake into vacuoles in vivo, isolated protoplasts were labelled with $^{32}P_i$ (S.A. = 15 MBq·μmol^{-1}; Amersham) and vacuoles were prepared by the fast method of Kaiser et al. (1982). This method yields intact vacuoles within less than 1 min.

Xylem sap collection. Xylem sap was isolated from 21-d-old barley plants grown in the presence of varying P_i concentrations. The plants were prepared and xylem sap isolated with a Scholander pressure chamber as described by Wolf and Jeschke (1987).

Infiltration of leaves and preparation of infiltrate. Ten-day-old primary leaves were detached and infiltrated in vacuo by immersion in a solution containing 100 mmol·l^{-1} sorbitol and 1 mmol·l^{-1} $CaCl_2$ (or Ca-lactate when chloride was to be determined). After the surface of the leaves had been carefully dried, infiltrate was extracted by centrifugation (1000·*g* for 2 min). To measure P_i uptake, 12 mmol·l^{-1} KH_2PO_4, pH 6.5 and 2 mmol·l^{-1} $KHCO_3$ were included in the solution used for infiltration. (In the colorimetric determination, the solution gave a reading corresponding to 11.3 mmol·l^{-1} P_i.) In these experiments, the infiltrated leaves were illuminated at a fluence rate of 8 W·m^{-2}. After specified times, infiltrate was prepared as described above. The calculation of apoplastic solute concentrations was based on the following relationships: 1 g of primary leaf tissue contains 80 μl of apoplast and may be infiltrated with 300 μl of solution (Pfanz 1987).

Uptake of P_i from the transpiration stream. Primary leaves were cut and immersed in solutions containing 1 mmol·l^{-1} $CaCl_2$ and various concentrations of KH_2PO_4 (KOH, pH 6.0). The leaves were illuminated for 4 h. The amount of P_i taken up by the leaves was determined. After feeding P_i, infiltrate was prepared as described above.

Transport of P_i into protoplasts and vacuoles. Uptake of P_i by protoplasts and vacuoles was measured by the method of Martinoia et al. (1987). The incubation medium used for measuring P_i uptake by protoplasts contained 0.6 mol·l^{-1} glycine betaine, 1 mmol·l^{-1} $CaCl_2$ and 10 mmol·l^{-1} 2-(N(morpholino)ethanesulfonic acid (adjusted to pH 6.0 with 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)). The incubation medium used for measuring P_i uptake by vacuoles consisted of 0.3 mol·l^{-1} sucrose, 0.2 mol·l^{-1} glycine betaine, 2 mmol·l^{-1} EDTA, 6 mmol·l^{-1} $MgCl_2$, 1 mmol·l^{-1} $MnCl_2$, 30 mmol·l^{-1} K-gluconate, 30 mmol·l^{-1} 4-(2-hydroxyethyl)-piperazineethanesulfonic acid, 0.2% (w/v) bovine serum albumin, 1 mmol·l^{-1} dithiothreitol and 10 mmol·l^{-1} ATP (when indicated). The pH of the medium was adjusted to 7.8 with Tris.

Phosphate uptake by protoplasts was initiated by the addition of protoplasts to the $^{32}P_i$-containing incubation medium and terminated by centrifugation of the protoplasts through a layer of silicone oil AR 200 (Wacker Chemie, München, FRG). Uptake by isolated vacuoles was started by addition of the suspension of vacuoles to a medium containing labelled P_i. Silicone oil and water (40 μl) were layered on top of the solution. Uptake was stopped by flotation of the vacuoles through the layer of silicone oil.

Measurement of oxygen evolution. Photosynthetic oxygen evolution by the leaves was measured with an air-phase oxygen electrode at saturating CO_2 (Delieu and Walker 1981). The irradiance was 300 W·m^{-2}.

Determination of P_i and of anions. Inorganic phosphate in leaves was extracted in 5–7% (v/v) perchloric acid. After neutralization of the solution with 3 mol·l^{-1} K_2CO_3, P_i was measured by the method of Fiske-Subbarow (1925). Phosphate in protoplasts and vacuoles was determined by a one-step spectrophotometric assay (Bencini et al. 1983). In the latter case, bovine serum albumin was omitted from isolation media. Alternatively, anion contents were determined by anion-exchange chromatography as described by Schröppel-Meier and Kaiser (1988a).

Results

Phosphate uptake into leaf cells. To estimate the P_i concentration in the apoplast, primary barley leaves were infiltrated with a P_i-free solution and infiltrate, termed intercellular washing fluid (IWF), was obtained from the leaves by mild centrifugation. Apoplastic P_i concentrations were between 0.5 and 2 mmol·l^{-1}. Calculations were based on an estimated apoplast volume of 80 μl per g leaf fresh weight (Pfanz 1987). There was the possibility that part of the P_i detected in the IWF may have been due to contamination by cytosolic and vacuolar material released from broken cells. However, measurements of the activity of cytosolic, chloroplastic and vacuolar enzymes in the IWF showed that contamination with intracellular material was well below 0.5% (data not shown).

Depending on growth conditions, the predominant apoplastic anion was either nitrate or chloride; their concentrations ranged from 3 to 25 mmol·l^{-1}. Even in the presence of excess P_i in the nutrient solution, for example

Table 1. Anionic composition of xylem sap isolated from barley plants which were grown in nutrient solutions with varying P_i concentrations. Barley plants (21 d old) were cut 1.5–2 cm above the root/shoot junction. The lower part of each plant was placed in a pressure chamber so that the shoot was sealed about 0.5–1 cm above the root/shoot junction. The roots were still immersed in the growing medium. Pressure was applied, xylem sap collected and analyzed. The data are means ± SD from $n=7$ (P_i-deficient plants), $n=8$ (plants in 40 mmol·l^{-1}) and $n=12$ measurements (control plants)

Anion	P_i concentration in the medium (mmol·l^{-1})		
	0	1.5	40
Cl^-	2.21 ± 0.87	1.50 ± 0.66	1.10 ± 0.47 mmol·l^{-1}
NO_3^-	5.51 ± 1.71	5.15 ± 1.96	4.88 ± 1.29 mmol·l^{-1}
PO_4^{3-}	0.04 ± 0.06	0.30 ± 0.09	1.12 ± 0.33 mmol·l^{-1}
SO_4^{2-}	0.35 ± 0.32	0.24 ± 0.11	0.18 ± 0.05 mmol·l^{-1}

100 mmol·l^{-1} KH_2PO_4, the P_i concentration in the apoplast did not exceed 3 mmol·l^{-1}. This indicates either efficient retention of P_i by the root system or efficient P_i uptake by the leaf tissue from the xylem, or both. Table 1 shows that the P_i concentration in the xylem sap was very low in plants grown in P_i-deficient medium. The concentration of P_i was higher in plants grown in 1.5 mmol·l^{-1} P_i, and was further increased in plants which were grown at elevated P_i levels (40 mmol·l^{-1}) in the nutrient solution; however the increase in the xylem sap (factor of 4) was much lower than the increase in the nutrient solution (factor of 27). Concentrations of other anions were much less affected. This shows that barley roots function as the main barrier to uptake when barley plants are exposed to increased P_i levels in the rooting medium.

To determine the capacity of the leaf cells to import P_i from the apoplast, two methods were employed: (*i*) Leaves were infiltrated with P_i-containing solutions and illuminated to avoid anaerobiosis of the infiltrated tissue. To limit the rate of photosynthesis, a low photon fluence rate was chosen. After an incubation of 2–30 min, IWF was obtained by centrifuging the leaves. The phosphate concentration in the IWF decreased linearly with time (Fig. 1). The rate of P_i uptake was 2.5 µmol·(*g* fresh weight)$^{-1}$·h^{-1} as calculated from the decrease in the P_i concentration in the IWF. (*ii*) Detached leaves were placed upright in solutions of increasing P_i concentrations in the light so that the cut ends were covered by solution. After 4 h, IWF was prepared from the leaves to determine the P_i concentration in the apoplast. The total amount of P_i taken up by the leaves was also determined and compared with the P_i concentration in the apoplast. Figure 2 shows that at low P_i concentrations in the feeding solution essentially all P_i which was taken up by the leaves and appeared in the apoplast was taken up by the cells. Apoplastic P_i levels remained largely constant although P_i in the feeding solution increased. Increasing P_i levels were recovered in the IWF only when the feeding solution contained more than 10 mmol·l^{-1} P_i. At a concentration of 20 mmol·l^{-1} P_i in the feeding solution, the rate of uptake was 4 µmol·g fresh weight^{-1}·h^{-1}.

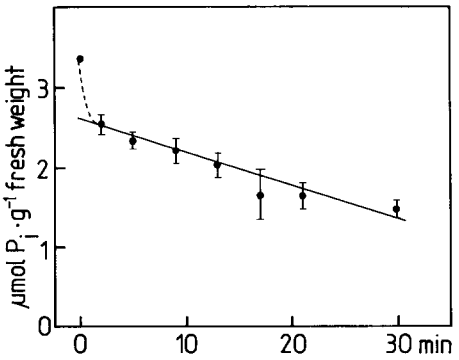

Fig. 1. Uptake of infiltrated Pi by barley leaves. Detached primary leaves were rapidly vacuum-infiltrated by immersion in a solution containing 12 mmol·l^{-1} P_i and illuminated at a low photon fluence rate. Infiltrate, termed intercellular washing fluid (IWF), was isolated at the times indicated and P_i was measured. The initial value gives the amount of P_i infiltrated into 1 g of leaf tissue as determined with 300 µl infiltration solution in the colorimetric assay (see *Material and methods*). The difference between the intersection of the line with the ordinate (time = 0 min) and the orignal concentration represents the dilution of the infiltrated P_i with apoplastic solution from the leaves. From the difference, a 1.25-fold dilution may be derived. Data are means ± SD ($n=4$)

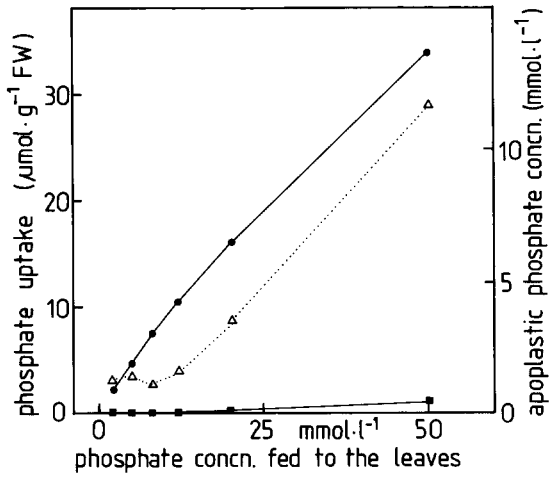

Fig. 2. Uptake of P_i into leaves and leaf cells of barley after feeding a solution containing P_i to the transpiration stream. For 4 h, detached leaves were placed in solutions of various P_i concentrations (*abscissa*) so that the cut ends were immersed in solution. The total amount of P_i taken up by the leaves (●–●) was determined by measuring the volume of the P_i solutions before and after incubation. The P_i in the IWF (■–■) was determined after rapid vacuum-infiltration with P_i-free solution and centrifugation of the leaves. 300 µl infiltrate were obtained per 1 g leaf fresh weight. The calculation of apoplastic P_i concentrations (△–△) was based on a ratio of 300 µl infiltrate per 80 µl apoplast

Both experimental approaches reveal that the leaf cells have a large capacity for P_i import from the apoplast. Most of the leaf P_i is compartmentalized in the mesophyll cells. When barley was grown in hydroponic

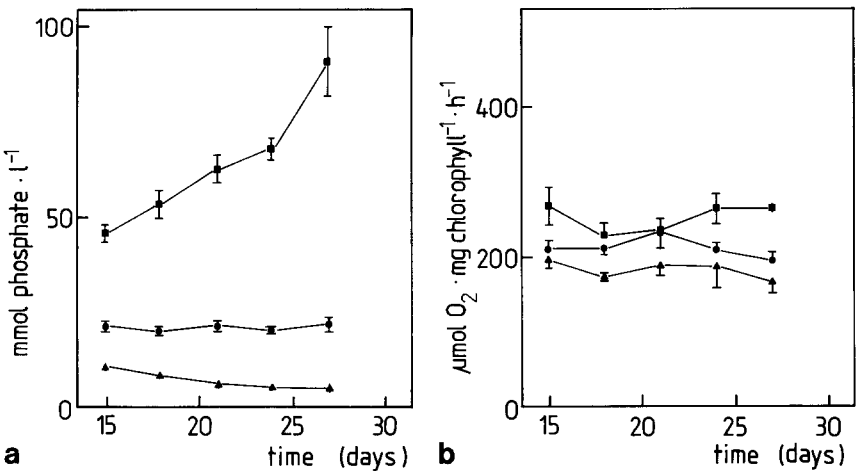

Fig. 3a, b. Dependence of P_i concentrations (**a**) and rates of light- and CO_2-saturated oxygen evolution (**b**) of leaves of barley plants on the P_i nutrition of the plants. Plants were grown on P_i-free nutrient solution (▲–▲) or in nutrient solutions containing 1.5 mmol·l^{-1} P_i (●–●) or 40 mmol·l^{-1} P_i (■–■). The data are means ± SE of 4–11 experiments. Photosynthesis was measured under light and CO_2 saturation at 20° C

culture under standard conditions (1.5 mmol·l^{-1} P_i in the nutrient medium), the concentration of P_i in epidermal protoplasts was only 2 mmol·l^{-1}, whereas 67 mmol·l^{-1} P_i was measured in mesophyll protoplasts (data not shown). Thus, mesophyll cells are sinks for external P_i.

Phosphate compartmentation in barley leaves. For the following experiments, barley was grown in the presence of high (40 mmol·l^{-1}) and normal (1.5 mmol·l^{-1}) concentrations of P_i or in the absence of P_i. In order to accelerate P_i depletion of the plants grown in the absence of P_i, seeds and primary leaves were removed 10 d after sowing. Figure 3 shows the changes in leaf P_i content in the period from 15 to 27 d after sowing. The average P_i concentration in the leaves decreased from 12 to 4 mmol·l^{-1} under P_i deficiency, remained at a constant level of 20 mmol·l^{-1} in control plants and reached concentrations as high as 90 mmol·l^{-1} in plants grown in excess P_i. Interestingly, photosynthesis was remarkably little affected by phosphate deficiency or excess phosphate (Fig. 3b). The highest rates of CO_2 fixation were observed in leaves of plants grown in excess P_i, but P_i deficiency did not decrease photosynthesis by more than 15% compared with plants grown in the presence of 1.5 mmol·l^{-1} phosphate, even when the leaf P_i concentration dropped in the deficient plants to levels as low as 4 mmol·l^{-1} (also compare Schröppel-Meier and Kaiser 1988b).

We isolated protoplasts and vacuoles to investigate the dependence of intracellular compartmentation of P_i on the growth conditions. Table 2 shows that the main portion of the cellular P_i was vacuolar P_i when the plants received sufficient P_i for normal growth. With 1.5 mmol·l^{-1} P_i in the nutrient medium, the vacuoles contained 87% of the total P_i of mesophyll protoplasts, and with 40 mmol·l^{-1} P_i available in the medium, this figure increased to 94%. Under condition of P_i starvation, however, the vacuolar P_i was only 45% of the total P_i, although the vacuolar volume exceeded the cytoplasmic volume by a factor of about 4.

Table 2. Dependence of the intracellular distribution of P_i in protoplasts on growth conditions. Barley was grown with and without P_i in the nutrient medium. Protoplasts and vacuoles were isolated from secondary leaves 21–23 d after sowing and P_i was measured. To calculate P_i contents (*A*), α-mannosidase activity was determined in the preparations of protoplasts and vacuoles and chlorophyll was measured in the protoplasts. The ratio of α-mannosidase activity to the chlorophyll content of the protoplasts was used to relate vacuolar P_i contents to the chlorophyll content of the protoplasts. The difference between vacuolar and protoplast P_i was attributed to the cytoplasm of the protoplasts. In different protoplast preparations, cytoplasmic P_i contents varied between 0.5 and 1.6 µmol·mg chlorophyll^{-1} in control plants, between 0.5 and 1.5 µmol·mg chlorophyll^{-1} in plants grown in 1.5 mmol·l^{-1} P_i, and between 0.7 and 2.0 µmol·mg chlorophyll^{-1} in plants grown in 40 mmol·l^{-1} P_i. Calculations of P_i concentrations (*B*) were based on a protoplast volume of 200 µl per mg chlorophyll, a vacuolar volume of 160 µl per mg chlorophyll and a cytoplasmic volume of 40 µl per mg chlorophyll. Mean values ± SE were calculated from measurements of three to six different experiments

A)

P_i content of nutrient solution (mmol·l^{-1} P_i)	P_i content (µmol P_i·mg chlorophyll^{-1})		
	Protoplasts	Vacuoles	Cytoplasm
0	1.9 ± 0.2	0.8 ± 0.2	1.0 ± 0.2
1.5	7.5 ± 0.6	6.5 ± 0.3	1.0 ± 0.3
40	24.6 ± 2.2	23.2 ± 1.8	1.4 ± 0.4

B)

P_i content of nutrient solution (mmol·l^{-1} P_i)	P_i concentration (mmol·l^{-1})		
	Protoplasts	Vacuoles	Cytoplasm
0	9.4 ± 0.9	5.2 ± 0.9	26.0 ± 4.8
1.5	37.4 ± 2.7	40.5 ± 2.0	24.8 ± 2.1
40	122.8 ± 11.1	144.7 ± 11.5	35.1 ± 9.4

Phosphate uptake by isolated protoplasts. Mesophyll protoplasts were isolated from leaves of plants which were either P_i-deficient or grown with 1.5 mmol·l^{-1} P_i in the

nutrient medium. They were then incubated with various P_i concentrations. Figure 4 shows uptake rates as a function of the concentration of external P_i. Uptake is biphasic, with a saturable component at low P_i concentrations and a non-saturable component at high P_i concentrations. A double-reciprocal plot of the uptake data revealed an apparent affinity (K_m) of the plasmalemma P_i transporter close to 100–150 $\mu mol \cdot l^{-1}$ P_i.

Table 3 shows uptake of P_i by mesophyll protoplasts in the presence of 1 $mmol \cdot l^{-1}$ P_i. This concentration is close to the concentration measured in the apoplast in vivo. When the plants used for protoplast isolation had been grown in the presence of excess P_i, uptake was less than half the uptake of protoplasts from plants grown in adequate P_i. Very similar observations were made in experiments with intact leaves. In infiltrated leaves from P_i-deficient plants, P_i was taken up at rates higher by a factor of 1.5–3 than in leaves from control plants (results not shown). However there was one important difference between experiments with leaves (Fig. 1) and isolated mesophyll protoplasts (Fig. 4): P_i uptake by the protoplasts was much lower than uptake obtained with leaves.

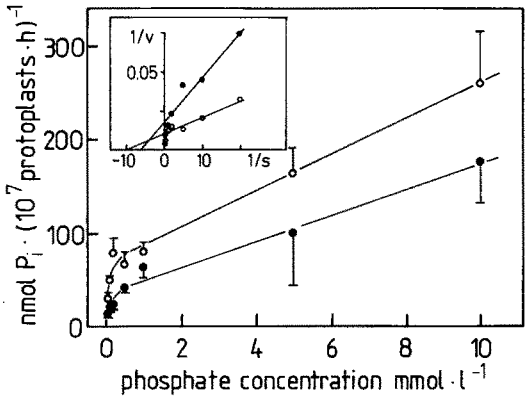

Fig. 4. Uptake of $^{32}P_i$ by isolated barley mesophyll protoplasts which were either isolated from P_i-fertilized (●–●) control plants or from P_i-starved plants (○–○). The data show the concentration dependence of P_i uptake. In the *insert*, the data are given as Lineweaver-Burk plot. The data are means ± SE of two to five experiments. 10^7 protoplasts of secondary leaves correspond to a volume of 35 µl. Multiplication of the given rates by a factor of 6 will approximate the rates per g fresh weight

Table 3. Uptake of P_i by protoplasts isolated from barley plants which were either P_i-deficient or grown with 1.5 or 40 $mmol \cdot l^{-1}$ P_i in the nutrient medium. Protoplasts were isolated from secondary leaves which were harvested 25 d after sowing and incubated with 1 $mmol \cdot l^{-1}$ $KH_2{}^{32}PO_4$, pH 7.8. 10^7 protoplasts isolated from secondary leaves contain 220 µg chlorophyll

P_i in the nutrient-medium ($mmol \cdot l^{-1}$)	Rate of P_i uptake ($nmol \cdot (10^7$ protoplasts$)^{-1} \cdot h^{-1}$)	
0	79 ± 9	(n = 3)
1.5	63 ± 11	(n = 5)
40	31 ± 5	(n = 4)

Transport of P_i into the mesophyll vacuole. To determine how fast P_i is distributed between the cytoplasm and the vacuole once it has entered the mesophyll, protoplasts were incubated with 10 $mmol \cdot l^{-1}$ $^{32}P_i$. Vacuoles were rapidly isolated (Kaiser et al. 1982) and analyzed for $^{32}P_i$ imported into vacuoles (Fig. 5). Inorganic phosphate appeared only slowly in the vacuole when protoplasts of control plants were incubated; the rate of import was much faster in protoplasts from P_i-deficient plants. This was only in part due to faster P_i uptake by P_i-deficient protoplasts. One can estimate the rates of P_i uptake from the cytoplasm into the vacuoles of isolated protoplasts using the data of P_i uptake by isolated protoplasts (Fig. 4), the specific activity of the incubation medium and the cytoplasmic P_i concentrations of the protoplasts (Table 2). Based on the radioactivity detected in the vacuoles after 90 min, uptake was 130 $nmol \cdot (10^{-7}$ vacuoles$)^{-1} \cdot h^{-1}$ in protoplasts from control plants and 240 $nmol \cdot (10^{-7}$ vacuoles$)^{-1} \cdot h^{-1}$ in protoplasts from P_i-deficient plants. In mesophyll cells which were grown in adequate or excess P_i, the P_i concentration was higher in the vacuole than in the cytoplasm (Table 2). Apparently, in this situation, P_i uptake into the vacuole is slow. In P_i-deficient cells, on the other hand, the vacuolar P_i concentration was much lower than the cytoplasmic concentration (Table 2). This turns vacuoles into efficient sinks for P_i, dependent on the electrochemical gradient (Kaiser et al. 1988).

Figure 6 shows uptake of P_i by isolated vacuoles in the absence (A) and in the presence of ATP (B). Without added ATP, rates of P_i uptake with vacuoles from control and from deficient plants were similar. ATP stimulated uptake particularly by vacuoles from P_i-starved

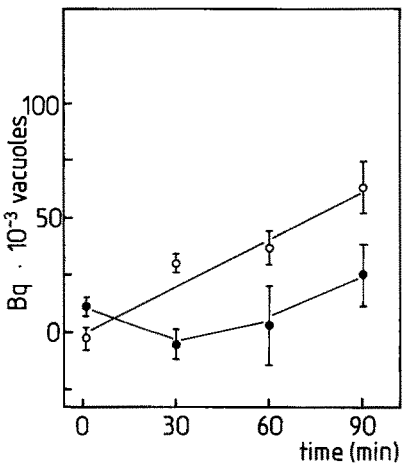

Fig. 5. Transport of P_i into the vacuoles of isolated barley mesophyll protoplasts. Protoplasts were incubated with 10 $mmol \cdot l^{-1}$ P_i which was labelled with ^{32}P. Vacuoles were isolated by a fast method and analyzed for radioactivity. ●–●, control plants; ○–○ plants grown in P_i-deficient medium. The points represent means ± SD of three to six measurements

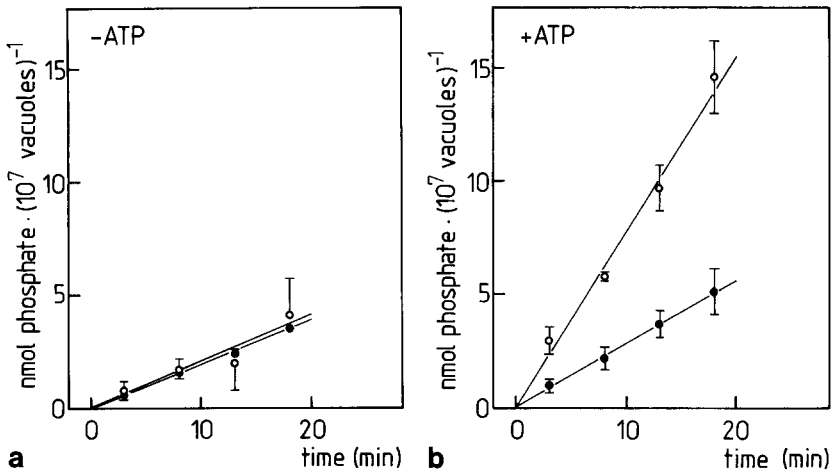

Fig. 6a, b. Time course of ATP dependence of P_i uptake by vacuoles isolated from control (●–●) or P_i-starved plants (○–○). The P_i concentration was 10 mmol·l^{-1} ($3 \cdot 10^5$ Bq per sample of 100 µl). **a** No ATP was added; **b** 1 mmol·l^{-1} ATP was included in the uptake solution. The data are means ± SE of three experiments with two to three replicates each. Multiplication by a factor of 6 makes the data comparable to the leaf data per g fresh weight

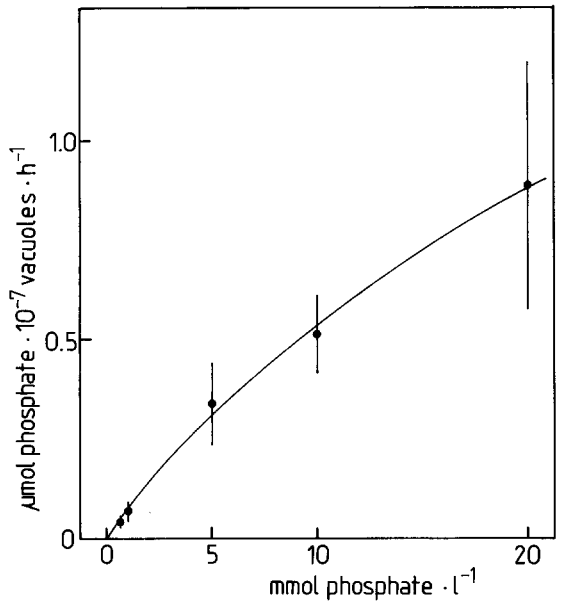

Fig. 7. Concentration dependence of uptake of P_i by isolated vacuoles. In contrast to the experiments shown in Figs. 5 and 6, the vacuoles were prepared from protoplasts isolated from primary leaves of barley plants grown under standard conditions. ATP was added to a final concentration of 10 mmol·l^{-1}. The size of vacuoles from primary leaves is larger than that of vacuoles from secondary leaves. A larger surface area of the tonoplasts explains the higher rates of uptake. The data may be directly compared with the leaf data per g fresh weight. Values are means ± SD ($n = 3$; each experiment representing a kinetic analysis with six time points)

plants, much less so by vacuoles which contained a high level of P_i (see Table 2).

Figure 7 shows the concentration dependence of P_i uptake by isolated vacuoles from normal plants. Saturation of uptake was not observed within the concentration range investigated. As uptake of P_i by vacuoles which were isolated from control plants was not much stimulated by ATP (Fig. 6), transport may be mediated by a facilitated diffusion mechanism along the electrochemical gradient.

Discussion

Photosynthesis is not much affected by P_i nutrition. Chlorophyll-related photosynthesis did no change much when the P_i content of the leaves was varied by a factor of 20. The relative insensitivity of photosynthesis to P_i deprivation during growth contrasts to results where a rapid inhibition of photosynthesis was seen after feeding mannose to intact leaves (Walker and Robinson 1978; Harris et al. 1983). Mannose is phosphorylated in the cytoplasm thereby effectively decreasing cytoplasmic P_i concentrations. The contrasting observations can be reconciled if it is assumed that P_i starvation primarily affects vacuolar P_i whereas mannose feeding causes sequestration of cytoplasmic P_i (Lee and Ratcliffe 1983; Rebeille et al. 1983). Obviously, the vacuoles stored P_i when it was available and released it on demand. The variability of vacuolar P_i levels is in remarkable contrast to the relative constancy of cytoplasmic P_i concentrations. The results bear witness to the role of the vacuole in maintaining cytoplasmic P_i homeostasis in mesophyll cells (see also: Foyer and Spencer 1986; Schröppel-Meier and Kaiser 1988b).

The relationship between the P_i and organic-phosphate pools. It should be noted that P_i homeostasis includes also phosphate esters. For chloroplasts, it is known that the total pool of P_i and organic phosphate esters is constant (for a review, see Heber and Heldt 1981). When organic phosphate esters accumulate in the chloroplasts during photosynthesis, P_i decreases. Similar relations hold also for the cytosol. In the present investigation, P_i was measured under conditions, where phosphate-ester accumulation did not significantly decrease cytoplasmic P_i levels. Phosphate is not only a key substrate in energy metabolism. It also plays a role in regulating enzymes. It is required for activation of ribulose-1,5-bisphosphate carboxylase (Heldt et al. 1978) and it inhibits ADP-glucose pyrophosphorylase (Preiss et al. 1967). At low chloroplast P_i levels, photosynthetic starch synthesis is activated (Heldt et al. 1977), and is sup-

pressed in relation to sucrose synthesis when P_i levels are adequate. To make such regulation independent of external factors, P_i fluxes across limiting membranes must be under control. Apparently, homeostasis is an expression of such control which is exerted at several levels. One of them is the restriction of P_i entry into the root system which prevents P_i flooding of the plant when this nutrient is abundantly available.

The role of the vacuole in regulating cytosolic P_i levels. When the root barrier is circumvented by feeding P_i to detached leaves via the transpiration stream, stomata close, thus restricting transpiration. This decreases P_i transport to the mesophyll (Harris et al. 1983; Dietz and Foyer 1986). The epidermis and the apoplast appear to be of little importance for the leaf P_i homeostasis. Excess P_i is transferred across the cytoplasm of the mesophyll cells into the vacuoles which accumulate P_i (Table 2). Accumulation is facilitated by an electrical potential across the tonoplast which favours anion uptake. It is all the more interesting that cytoplasmic P_i homeostasis can be maintained under P_i starvation also by export of P_i from the vacuole into the cytoplasm. Table 2 shows that such export is directed against a concentration gradient and, presumably, also against an electrical potential. The high cytosolic concentration of proteins which carry a net negative charge produces a diffusion potential across the tonoplast even if there is no active electrogenic transport of positively charged ions into the vacuole. As a matter of fact, an H^+-translocating ATPase and a pyrophosphatase are known to pump protons into the vacuole, generating not only a proton gradient but also an electrical potential across the tonoplast (for a review, see Sze 1985).

Plants depleted of P_i such as spinach (Dietz 1989) and barley (data not shown) rapidly accumulate P_i in the leaves once P_i is added to the nutrient solution. Concentrations of P_i in the leaves may be as high as 120 $mmol \cdot l^{-1}$ within 3 d after transfer from P_i deficient medium to a nutrient solution containing only 1.5 $mmol \cdot l^{-1}$ P_i. Obviously a transport system with large capacity for P_i uptake was induced in the root system when the plants were deprived of P_i and catalyzed a rapid accumulation of P_i in the leaves once the P_i availability was improved. The increase in the P_i concentration of the leaves had remarkably little effect on photosynthesis. Together with the result of Fig. 5, these observations show that the tonoplast has a large capacity for P_i transport. This capacity can also be demonstrated with isolated vacuoles. There was a large difference in ATP-driven P_i uptake between vacuoles isolated from control and from P_i-deficient leaves. The reason for this difference is not known.

Recently, two types of ion channel have been characterized in the tonoplast of storage tissue of beet roots (Hedrich and Neher 1987) and in tonoplast membranes of cells of other tissues (Hedrich et al. 1988). These channels mediate anion and cation fluxes and may also transport P_i in a manner dependent on the electrochemical potential gradient. However, there was a large difference in ATP stimulation when P_i uptake was studied in vacuoles isolated from P_i-deficient or from control leaves. This could indicate the involvement of a secondary activated-transport system.

Phosphate homeostasis in the cytoplasm requires export of P_i from the vacuole when the cytoplasmic P_i concentration decreases. Efflux of P_i was low both in the presence and in the absence of ATP in the medium (result not shown). The regulation of efflux as part of the mechanism to maintain the cytoplasmic P_i homeostasis is not yet understood.

Transport of P_i across the plasmalemma. Interestingly, the rates of P_i uptake by protoplasts isolated from P_i-deficient leaves were higher than rates of uptake by protoplasts from control plants (Fig. 4). An increased rate of P_i uptake has also been observed under P_i deficiency in roots, algae or higher plants (Humphries 1951; Falkner et al. 1980; Drew et al. 1984; Ullrich-Eberius et al. 1984). It should be mentioned that cytoplasmic P_i concentrations were similar in P_i-starved leaves and in leaves of plants which were fertilized with P_i. They were higher than external concentrations (Table 2). Moreover, the membrane potential of the plasmalemma is positive outside so that P_i must be transported not only against a concentration gradient, but also against a membrane potential. Ullrich-Eberius et al. (1984) and Goldstein and Hunziker (1985) have suggested that uptake of inorganic P_i is facilitated by the proton motive force generated by the H^+-ATPase of the plasmalemma.

A significant difference in transport rate was observed between intact leaves and isolated mesophyll protoplasts. Two explanations are possible: (*i*) the P_i transporter system of protoplasts is damaged during protoplast isolation by the action of hydrolytic enzymes; (*ii*) specialized cells bordering the vascular bundles are equipped with transporters, and the isolation procedure used for obtaining mesophyll protoplasts discriminates against these cells. Inorganic phosphate could be distributed from the specialized cells into the mesophyll by symplastic connections.

In this context, it is of interest that pyranine, a large trivalent anion, has been observed microsopically to be readily taken up from the transpiration stream into cells closely connected to the xylem, when it is fed to leaves through the petiole. After prolonged periods of feeding, mesophyll protoplasts contained pyranine which could easily be detected by its fluorescence. Isolated mesophyll protoplasts, on the other hand, proved to be unable to take up pyranine from the surrounding solution (data not shown).

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